XXV. THE INORGANIC PHOSPHATE AND A LABILE FORM OF ORGANIC PHOSPHATE IN THE GASTROCNEMIUS OF THE FROG.

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In a study of the significance of phosphorus in muscle contraction (which will be reported separately) some observations were made which throw a doubt on the chemical results of some earlier workers. Evidence is given in this paper to show that the supposed inorganic phosphate of muscle is in certain conditions mainly organic phosphate of a very labile nature, which is so unstable in acid solution that it is hydrolysed during the estimation of the inorganic phosphate by the methods of Neumann, Embden [1921] or Briggs [1922]. Estimations performed in neutral or slightly alkaline solution, such as the Bell-Doisy method [1920], or precipitation by magnesia mixture, give results approximating to the truth, provided the muscle extract has not previously been exposed to the action of acid.

Briggs' method itself can be used to demonstrate the existence of this unstable phosphoric ester. Details of the method are given elsewhere: it suffices for the moment to say that after the addition of the appropriate reagents to a solution containing phosphate a blue colour develops, which rises to a maximum in about 30 minutes, after which time colour comparisons are usually made. It is easy to show that the rate of development of this blue colour follows a simple exponential law,

$$\text{intensity at time } t = c = P\,(1 - e^{-kt}),$$

where P is the final colour which measures the phosphate. The constant k has the value 0·12 (time measured in minutes). It follows mathematically (and can be proved experimentally) that in comparing two quantities of inorganic phosphate the ratio of the colour intensities will be the same at whatever time the comparison is made. If the colour ratio be plotted against time the result is a straight line parallel to the time axis (lines AB and CD in Fig. 1). A deproteinised extract from a dead muscle (killed by warming to 35° for 20 minutes) compared in this manner against an inorganic phosphate solution gives also a straight line, and is indistinguishable in this respect from an inorganic phosphate solution. When however a similar extract from a resting muscle is compared against an inorganic standard a different result

is obtained. The colour development begins by appearing equivalent to a very weak standard, but rapidly overtakes a much stronger standard. The result is a curve such as the curves R and R' in Fig. 1.

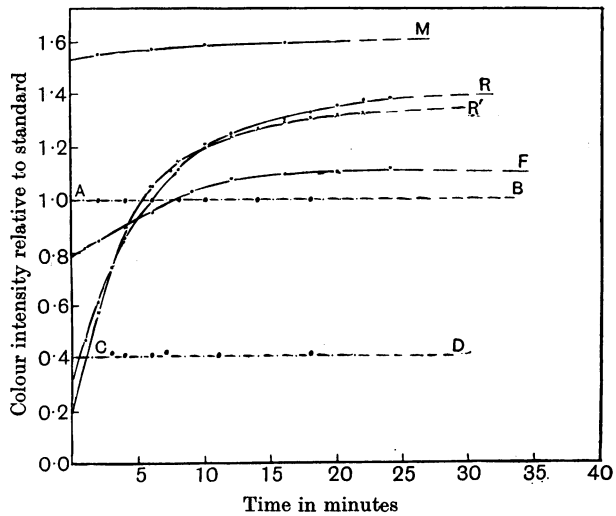

Fig. 1. The inorganic phosphate solution which gave the line AB contained 0·1 mg. of phosphorus. Another solution containing 0·04 mg. gave the parallel line CD. The remaining curves were given by muscle filtrates corresponding in each case to 160 mg. of muscle. The lines represent the colour intensity at different times relative to the standard solution which gave the line AB. (R and R', resting; F, fatigued; M, in rigor.)

So complex are the conditions of colour development that little importance would attach to this fact were it not that a fatigued muscle shows this effect to a much less extent (curve F), whilst as has been said already, a muscle in rigor mortis shows no such effect at all (line M). Of the possible explanations which present themselves, the only one which survives the test of experiment is that there is an organic phosphate breaking down during the course of the estimation, causing the colour development curve to be pushed higher and higher. That this substance is completely destroyed in 20 minutes or so is shown by the curves in Fig. 2. In this type of experiment two portions of a resting muscle filtrate are treated simultaneously with Briggs' acid molybdate solution, but in one case the reducing solution is not added until 30 minutes later. In the latter case the colour production curve runs strictly parallel with an inorganic standard. The unstable organic phosphate has already broken down.

As to the nature of this unstable compound we have no evidence, save that it is concerned in muscular activity, and its disappearance coincides with fatigue. The name "phosphagen" suggests itself. "Phosphagen" might possibly be a phosphoric ester of glycogen, for traces of glycogen are present in trichloroacetic acid extracts of muscle. It is also possible that it may be the substance postulated by Meyerhof as a precursor for both lactic acid and "lactacidogen."

When Briggs' method is used to estimate the true inorganic phosphate, all that is necessary is to make colour comparison with the standard at frequent intervals, and obtain a curve such as those in Fig. 1. By extrapolating this curve backwards to zero time a value is obtained which represents the colour ratio which would have been given at the end of 30 minutes had the "phosphagen" been stable. The difference between this true value and that obtained after 30 minutes, measures the "phosphagen" in terms of the inorganic phosphate formed by its decomposition.

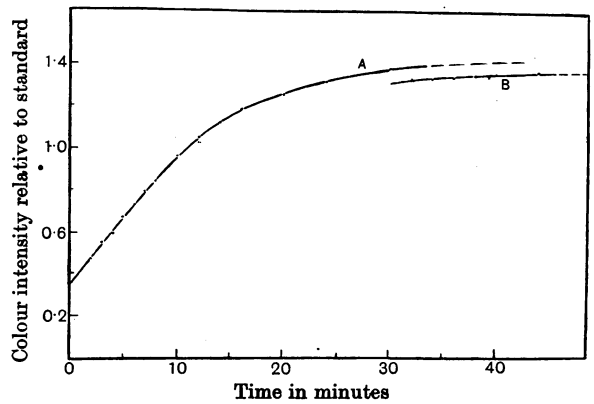

Fig. 2. The curve *A* represents the colour intensity at different times (relative to an inorganic standard started off at the same moment) given by an extract from a resting muscle, when the "inorganic" phosphate is estimated by Briggs' method. If the reducing agent is added 30 minutes after the acid molybdate reagent the colour production curve (*B*) is such as would be given by true inorganic phosphate. The "phosphagen" is already broken down. (The starting-point of the curve *A* is a measure of the true inorganic phosphate, whilst the height to which the curve rises is a measure of the "phosphagen.")

Table I. *Showing the rapid breakdown of "phosphagen" in acid solution.*

A fresh trichloroacetic acid filtrate from a resting muscle was estimated for inorganic phosphate and "phosphagen." Part of the filtrate was neutralised with $NaHCO_3$ and the remainder not treated. Both portions were incubated for an hour at 27° and the estimations were repeated on each. Results given in mg. of phosphorus per 100 g. of muscle.

	Inorganic *P*	"Phosphagen"	Increase in inorganic *P*	Decrease in "phosphagen"
Initial	27	57	—	—
After 1 hour at 27°	51·5	33	24·5	24
Incubated in presence of $NaHCO_3$	30·5	50	3·5	7

We have used 4 % trichloroacetic acid as a deproteinising agent, and have found that even in this solution the unknown substance disappears in a day or two. So rapid is this breakdown in the first hour or so, that special precautions must be taken to perform estimations as soon as possible after the removal of protein. Alternatively the extract can be neutralised with sodium bicarbonate, which renders the "phosphagen" less liable to breakdown on standing (Table I).

The stability of "phosphagen" in neutral solution led us to try the estimation of inorganic phosphate by the Bell-Doisy method, which is performed

in a mildly alkaline solution. The results (see Table II) agreed closely with the "corrected" values obtained by Briggs' method. In passing it should be mentioned that Bell and Doisy probably found traces of "phosphagen" in blood, for they state that "the inorganic phosphate must be determined in the trichloroacetic acid filtrate as soon as possible after filtering since the acid hydrolyses the organic phosphorus on standing, giving too high results. After standing 24 hours the values for inorganic phosphate and the total acid-soluble phosphorus are nearly identical." In the case of blood however the discrepancy appears to be a small one—not more than about 10 % of the value quoted for inorganic phosphate. In resting muscle extracts we have a discrepancy of the order of 300 %.

Estimation of the inorganic phosphate with magnesia mixture confirmed our belief that we were dealing with an organic phosphate unstable in acid. Although this method is notorious for giving high results (on account of the simultaneous precipitation of calcium and organic matter), our figures indicate that a resting muscle contains not more than 40 mg. % of inorganic phosphorus, less than half the figure given by the method of Embden (or any method involving the use of mineral acids).

Table II. *The "inorganic phosphate" of the frog's gastrocnemius, showing the falsely high values given by the Briggs and Embden methods, particularly for resting muscles.*

Exps. A, D, E and K were performed on batches of 6 to 8 frogs. In Exps. E, F and G the left gastrocnemius was used as a resting control on the right, which was stimulated through the nerve for 2 to 5 minutes with a supermaximal stimulation. The Briggs and Embden methods give high resting values which fall in fatigue. The other methods give low values which rise considerably in fatigue. All methods give the same high value for rigor.

		"Inorganic P" in mg. per 100 g. of muscle				
Reference		Embden	Briggs	Briggs corrected	Bell-Doisy	Magnesia mixture
Resting	A	92	87	—	—	—
	B	—	83	—	22	—
	C	—	91	18	—	—
	D	—	88	—	—	40
	E	95	86	—	—	—
	F	—	87	33	—	—
	G	—	84	28	27	—
	K	—	90	22	28	12
Fatigued	E	75	72	—	—	—
	F	—	73	56	—	—
	G	—	68	45	52	—
Rigor	H	—	105	102	—	—
	I	—	96	102	—	—
	K	—	104	90	108	103

EXPERIMENTAL.

The muscles were killed by immersion in liquid air and ground up with 4 % trichloroacetic acid in a mortar. The fluid was washed into an accurately calibrated cylinder and the volume made up with 4 % trichloroacetic acid to 4 or 5 cc. per 100 mg. of muscle. It was found essential to keep the temperature down to 0° until the actual estimation was performed; moreover the

fluid was filtered within 10 minutes of the maceration. Contrary to the results of certain other workers we have found it quite unnecessary to allow the extraction to continue overnight: extraction in 3 minutes was almost as complete as in 20 hours.

A quantity of filtrate calculated to contain about 0·1 mg. of phosphorus (inorganic plus "phosphagen") was diluted in a 15 cc. graduated flask to exactly 12 cc. An inorganic standard of equal or less strength contained in a similar flask was treated with the same amount of 4 % trichloroacetic acid and similarly diluted. The two reagents were added[1], and the solutions after being mixed were poured directly into the colorimeter cups. Readings could in this way be made within the first minute of the period of colour production. It is essential in following the colour production curves that the unknown and standard should be started off simultaneously and should be at the same temperature.

Where the Embden technique was used Embden's directions were followed in every detail, save that trichloroacetic acid was used to deproteinise the muscles. This is a simpler method and gives the same results as the Schenck method recommended by Embden.

The Bell-Doisy method was modified slightly to meet our special requirements. Instead of allowing 5 minutes for their acid-molybdate-quinol solutions to act before the addition of the sulphite-carbonate reagent, we reduced the time to about 1 minute for both muscle extract and inorganic standard.

The magnesia mixture used contained magnesium citrate instead of the chloride. This is supposed to give truer values.

Discussion.

We have found that the true inorganic phosphate content of a resting frog's gastrocnemius is of the order of 20 to 25 mg. of phosphorus per 100 g. of muscle. Estimations performed by the Briggs or Embden method give results of the order of 90 to 100 mg. per 100 g. in our hands (Embden's own figures are rather higher). This discrepancy of 70 mg. per 100 g. is attributable to a phosphoric ester which is very unstable in acid solution, and which is estimated as inorganic phosphate by the above methods. We have called this new compound "phosphagen."

In rapidly induced fatigue the true inorganic phosphate content is doubled at the expense of the "phosphagen," but considerably more "phosphagen" disappears than is necessary to account for the rise in inorganic phosphate. It is obvious that a method which estimates both inorganic phosphorus and "phosphagen" together should show a fall as a result of fatigue. We have found such a fall by both Embden's and Briggs' methods, though Embden

[1] Solution A: 5 % ammonium molybdate in 5·4 *N* sulphuric acid. Solution B: 0·5 % quinol in 20 % sodium sulphite. Solution B reduces the phosphomolybdate formed by the interaction of solution A with the inorganic phosphate.

himself claims to have found a rise [Embden *et al.*, 1922, 1925, 1926]. The figures quoted by Embden are so high, for both resting and fatigued muscles, that they cannot refer to the true inorganic phosphate.

When a muscle goes into rigor the "phosphagen" disappears entirely, and the inorganic phosphate rises to about four times the resting value. Here the "phosphagen" is more than accounted for by the inorganic phosphate which appears.

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